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Enviro Control, Inc, Rockville, MD

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ACUTE TOXICITY TESTING CRITERIA FOR CHEMICAL SUBSTANCES

Contract No. W 2227-NA5X

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ABSTRACT

This report addresses the rationale, considerations, and limitations of acute toxicity testing. General procedures are described for acute tests including lethality studies in oral, dermal, and inhalation toxicity, and irritation studies in dermal and eye toxicity, phototoxicity and skin sensitization. Recommendations are given for the acute toxicity tests which may be used to evaluate the risks associated with the manufacture and processing of chemical substances. Suggested minimum protocols are offered including the choice of test animals, dosage levels, laboratory practices, animal diets, and necropsy requirements. A method is suggested for interpreting the results of these tests and extrapolating to some guidelines for a safety factor in human exposure.

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FORWARD

This study was conducted for the Premanufacturing Review Division, Office of Toxic Substances, Environmental Protection Agency, to collect data and develop procedures for the assessment of toxicity as an integral part of the risk assessment scheme for new chemical substances. This publication, which focuses on acute toxicity assessment, was directed primarily toward the initial screening operation of chemical substances.

While studies on acute toxicity have appeared in the literature, this is the first report useful for the evaluation of the risks posed by new chemical substances to human health and the environment. Although this study focuses on new chemical substances, it is important to note that the procedures developed herein may have direct application to all areas of environmental health analysis.

This document was published with the expert advice of Dr. Peter Voytek, Office of Toxic Substances. Further information can be obtained from the Premanufacturing Review Division; telephone: 202-426-2601; address: Office of Toxic Substances (TS-794), U.S. Environmental Protection Agency, 401 M Street, SW, Washington, D.C. 20460.

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SECTION 1

INTRODUCTION

This report includes various suggestions for the collection and utilization of acute toxicity data in the screening of chemical substances.

Acute toxicity studies include lethality, irritation, sensitization and corrosion testing. Acute lethality protocols usually involve exposing an experimental animal to a single dose of a test substance for 24 hours or less. The animal is then observed for up to 14 days after exposure (1). The results may be expressed as a simple mortality response (death or no death) over a broad range of dose levels which are reported as mg test substance per kg body weight of experimental animal. These dose levels and associated mortality responses are then used to extrapolate the LD_{50} (lethal dose for 50% of a group of test animals) for the test substance. Specific toxic responses can also be measured and used to determine another useful statistic--the TD_{50}. This is the dose at which 50% of all test subjects develop a given toxic response such as tremors, erythema, convulsions, loss of consciousness, or malfunction of a specific organ system.

Acute irritation, sensitization, and corrosion tests are used to assess a chemical's potential for injury to specific tissue, usually the eye or skin. Interpretation of test results is more complex than for lethality testing and may include complex systems for grading toxic responses. Because death is not expected to result from these studies, LD_{50}'s are not calculated.

It would be of obvious benefit to be able to use the results derived from one type of acute test to predict the level of toxicity that would be seen for that same compound using the same animal model in another type of acute test. A major impediment to the feasibility of such correlations is that the toxicity data most commonly available for any given compound are usually collected for two different animal species - an oral LD_{50} in the rat and a dermal LD_{50} in the rabbit. However if both oral and dermal LD_{50}s are available in the same animal species, even when the data has been collected by two different laboratories, there are some potentially useful generalizations to be made. Table 1 shows the oral and dermal LD_{50}s where both are available for the same animal species for 51 compounds using 4 animal species in 57 tests (2). Dermal LD_{50}s are higher than, or at least equal to, oral LD_{50}s in about 88% of the cases. In every case but one, hexabutyl-distannoxane tested in the rat, the oral and dermal LD_{50}s are within one order of magnitude of each other. Data such as these suggest that substances which show toxicity upon dermal application will probably be as toxic or more toxic when administered orally. This is generally attributed to the more rapid systemic absorption of ingested substances.

TABLE 1

COMPARISONS OF ORAL AND DERMAL LD_{50}s

WITHIN THE SAME ANIMAL SPECIES

CHEMICAL	SPECIES	ORAL LD_{50} mg/kg	DERMAL LD_{50} mg/kg	DERMAL LD_{50}/ ORAL LD_{50}
ACROLEIN	RABBIT	7	562	80.29
ACRYLONITRILE	RABBIT	93	250	2.69
AMMONIUM, (2-CHLOROETHYL) TRIMETHYL-CHLORIDE	RABBIT	150	232	1.55
ANILINE	RAT	440	1400	3.18
p-CHLORO-ANILINE	RAT	420	3200	7.62
NITROBENZENE	RAT	640	2100	3.28
BENZENETHIOL	RAT	46	300	6.52
4,4' - BIPYRIDINIUM, 1,1'- DIMETHYL-DICHLORIDE	RAT	57	80	1.40
2-ETHYLBUTANOL	RABBIT	1200	1260	1.05
t-BUTYLHYDROPEROXIDE	RAT	406	790	1.95
CARBAMIC ACID METHYL -, 1-NAPHTHYL ESTER	RABBIT	710	2000	2.82
CARBON TETRACHLORIDE	RAT	2800	5070	1.81
m-CRESOL	RAT	242	620	2.56
o-CRESOL	RAT	121	1100	9.09
p-CRESOL	RAT	207	750	3.62
4,6 - DINITRO-o-CRESOL	RAT	10	200	20.00
p-DIOXANE	RABBIT	2000	7600	3.80
HEXABUTYL - DISTANNOXANE	RAT	87	11,700	134.48
4-METHYL -6- PYRIMIDINYL PHOSPHOROTHIOATE	RAT	2000	8000	4.00
1,2 - DIBROMO - ETHANE	RAT	108	300	2.78
	RABBIT	55	300	5.45
2-BUTOXY-ETHANOL	GUINEA PIG	1200	230	0.19
2-(2-BUTOXY-ETHOXY) ETHANOL ACETATE	RABBIT	2600	15,000	5.77
2-CHLORO-ETHANOL	RAT	58	84	1.45
2-ETHOXY-ETHANOL	RABBIT	3100	3500	1.13

TABLE 1 (CONTINUED)

CHEMICAL	SPECIES	ORAL LD_{50} mg/kg	DERMAL mg/kg	DERMAL LD_{50}/ ORAL LD_{50}
2-(2-ETHOXYETHOXY) - ETHANOL	RAT	6500	6000	0.93
2'-HYDROXY - 2,4,4' - TRICHLORODIPHENYL ETHER	RAT	3700	9300	2.51
N,N - DIMETHYL - FORMAMIDE	RAT	2800	5000	1.79
N'-(4-CHLORO-o-TOLYL)-N,N-DIMETHYL-FORMAMIDINE	RAT	170	4000	23.53
	MOUSE	160	225	1.41
	RABBIT	625	640	1.02
FORMIC ACID ETHYL ESTER	RABBIT	2075	20,000	9.64
METHYL-HEPTANETHIOL	RAT	85	1954	22.99
2-ETHYL-1,3-HEXANEDIOL	RABBIT	2600	2000	0.77
4-METHYL-3-PENTEN-2-ONE	RABBIT	1000	5990	5.99
PHENETHYL ALCOHOL	GUINEA PIG	400	5000	12.5
PHENOL	RAT	414	669	1.62
2-sec-BUTYL-4,6-DINITROPHENOL	RAT	25	80	3.20
4,4' - ISOPROPYLIDENEDI-PHENOL	RABBIT	2230	3000	1.35
PENTACHLORO-PHENOL	RAT	50	105	2.10
PHOSPHONIC ACID (2,2,2-TRICHLORO-1-HYDROXYETHYL)-DIMETHYL ESTER	RAT	450	2000	4.44
	RABBIT	1450	5000	3.45
PHOSPHORIC ACID 1,2-DIBROMO-2,2-DICHLORO-ETHYL DIMETHYL ESTER	RAT	250	800	3.20
S-((5-METHOXY-2-OXO-1,3,4-THIADIAZOL-3 (2H)-YL) METHYL) O,O-METHYL PHOSPHORO-DITHIOATE	RAT	25	20	0.80
	RABBIT	63	375	5.95
PHOSPHORODITHIOIC ACID, S-((2-ETHYLTHIO)ETHYL) O,O-DIMETHYL ESTER	RAT	25	179	7.16
PHOSPHOROTHIOIC ACID, O,O-DIETHYL O-(2-ISOPROPYL-6-METHYL-4-PYRIMIDINYL ESTER)	RAT	76	455	5.99
PHOSPHOROTHIOIC ACID, O,O-DIMETHYL ESTER, O,O-DIESTER WITH 4,4'-THIODIPHENOL	RAT	1000	1370	1.37

TABLE 1 (CONTINUED)

CHEMICAL	SPECIES	ORAL LD_{50} mg/kg	DERMAL mg/kg	DERMAL LD_{50}/ ORAL LD_{50}
PHTHALIC ACID, BIS (2-ETHYL-HEXYL) ESTER	RABBIT	34	25	0.74
POLYPROPYLENE GLYCOL MONO-BUTYL ETHER	RABBIT	23,900	21,000	0.88
1,2-DIBROMO-3-CHLORO-PROPANE	RABBIT	180	1400	7.78
1,2-EPOXY-PROPANE	GUINEA PIG	690	8640	12.52
TOXAPHENE	RAT	40	600	15.00
2-CHLORO-4-ETHYLAMINO-6-ISOPROPYLAMINO-S-TRIAZINE	RABBIT	750	7500	10.00
4-UNDECANOL, 7- ETHYL-2-METHYL-,HYDROGEN SULFATE, SODIUM SALT	GUINEA PIG	650	650	1.00
2,6 - XYLENOL	MOUSE	980	920	0.94
	RABBIT	700	1000	1.43

Acute toxicity testing is valuable in risk assessment in these ways:

- as a screening procedure to help identify substances of such low toxicity that extensive further acute testing is not justified;

- as a method to indicate specific toxic effects of a substance which might be associated with either a single massive exposure or with frequent use at a high level of exposure.

- as a range finder for studies by producing data concerning lethal levels, signs of intoxication, and possible target organ effects.

- as an aid in the design of appropriate clinical management programs for individuals involved in the misuse of or an accident with a chemical substance.

- as a means to develop an index of the relative hazard of acute exposure to various substances by comparing LD_{50}s and the slopes of their dose-response curves.

SECTION 2

TYPES OF ACUTE TOXICITY TESTS

ACUTE ORAL TOXICITY

Acute oral toxicity studies are designed to investigate the qualitative and quantitative nature of the toxic effects from a single oral exposure to a large dose of the chemical substance in question. The doses are selected to provide data sufficient to estimate the LD_{50} and to determine the slope of the dose-response curve. The numbers of test animals per dosage level should be sufficient to make these statistical analyses according to a particular method of evaluation. The test animals should be characterized as to species, strain, and physiological and morphological characteristics. There is no standardized animal that is suitable for all acute oral tests. It is extremely important that the test animals be randomly selected for the dose level groups.

The test substance, if it is not a liquid, should be administered in an appropriate carrier which facilitates absorption.

It is essential to note carefully all toxic signs including type, time of onset, severity, and duration. The time at which deaths occur or signs of toxicity appear is important, particularly if there is any tendency for deaths to be delayed. Observations of the animal should continue until signs of toxicity are absent in surviving animals. A 14-day observation period is sufficient for most compounds. At that time survivors should be sacrificed and submitted to a gross pathologic examination.

Ideally, to assess potential health hazards to humans, toxicity studies should be conducted only in those species of animals whose metabolism of the test substance is similar to that of humans. Since comparative metabolism is unknown for most new substances, and since studies in humans are rarely feasible at this stage, rodents make good initial test subjects. While extrapolation of the results to humans may not always be valid, the correlation is reasonably good for single oral doses. Acute toxicity testing can be conducted in several species if necessary. A similar degree of toxicity in several species would suggest that toxicity to humans might be comparable; marked variation in the responses of different species necessitates the assumption that humans are at least as sensitive as the most sensitive species studied.

It is suggested that these tests be performed on two species of animal-the albino rat, and the albino mouse. All female animals should be nulliparous. The minimum sample number and dosage schedule should be:

- 5 male and 5 female animals of each test species at each of five dose levels - preferably to include 2 levels above and 2 below the

expected LD_{50}. A suggested dosage schedule is: 5000 mg/kg, 500 mg/kg, 50 mg/kg, and 5mg/kg.

ACUTE DERMAL TOXICITY

The ability of some chemicals to penetrate either intact or abraded skin and produce systemic toxicity has been well established. A test for acute dermal toxicity should evaluate the potential for systemic toxic effects of chemicals expected to come in contact with the skin. This is done by determining the LD_{50} of a single dermal exposure to the compound in question by the animal test species. Since this LD_{50} will be used in hazard evaluation, the test conditions should be related to the anticipated human exposure, if at all possible.

The albino rabbit is the animal most frequently used in assessing dermal toxicity and the most commonly used procedure is the method of Draize.(3) It is also suggested that other animal species, such as mice, rats, guinea pigs, or dogs, be used in addition to rabbits. The rabbit appears to be exquisitely sensitive to dermal insult, and thus the elicited reactions may not be valid for humans. The skin of guinea pigs has permeability characteristics more like those of humans. The albino rat is somewhat less reactive than the rabbit, and more reactive than the guinea pig or humans. The rat is a preferred species for dermal lethality testing because it is the model used most often for acute oral studies and as well as for other types of toxicological studies.

Shortly before testing, fur from the trunks of healthy animals should be clipped so that no less than 10% of the dorsal body surface area is available for application of the substance. Care should be taken to avoid abrading the skin, since this would alter its permeability. However, a dermal LD_{50} for an animal with abraded skin may be desired since the human skin which may eventually be exposed to the test substance will not necessarily be intact.

For some applications, it may be appropriate or necessary to use a vehicle. If such is the case, any effect of the vehicle on the penetration of the test substance should be established. The test dose must remain in contact with the skin throughout the exposure period, usually 24 hours.

At the end of the exposure period, any residual material is gently removed with a gauze compress, the exposed area is examined, and any lesions are noted. The animals should be examined at least once daily for 14 days for signs of systemic toxicity and localized dermal reactions. All animals that succumb or are moribund are necropsied. At the end of the 14-day observation period, all survivors are subjected to a thorough clinical examination, including examination of the exposed area of skin. They are then sacrificed and necropsied. The degree of skin irritation, signs of intoxication, changes in body weight, mortality, and gross pathological findings as a function of dose and time are noted.

The method of calculating the acute dermal LD_{50} is the same as that for the acute oral LD_{50}. Both of these LD_{50}'s are convenient for estimating acute toxic hazard. Although there is always risk in extrapolation from animals to humans, it is usually safe to presume that substances with lower dermal LD_{50}s in animals will be potentially more toxic to humans than those with higher LD_{50}s.

It is suggested that these tests be conducted on two species of animal-the albino rabbit and the albino rat. Female animals should be nulliparous. The following sample numbers and dosages are recommended:

- 2 male and 2 female animals of each test species at each of five dose levels - preferably to include 2 levels above and 2 below the expected LD_{50}. A suggested dosage schedle is: 5000 mg/kg, 500 mg/kg, 50 mg/kg, 5mg/kg, and 0.5 mg/kg.

ACUTE INHALATION TOXICITY

The respiratory tract is particularly vulnerable to many substances since it is generally less protected than most body systems. Moreover, it can be subjected to injury not only when a toxicant enters the body through the respiratory tissues, but also, in some instances, when a toxicant leaves the body via the respiratory tract after having gained entry by a different route. Consequently, injuries to the lung and other body tissues resulting from inhaled toxic substances can have numerous ramifications depending on the degree of toxicity of the substance, the concentration and duration of exposure, and the existence of an immediate or latent effect. The anatomy and physiology of the respiratory tract can have great influence on the toxicity of inhaled vapors, gases, and particularly, inhaled particles.

Single, high concentration inhalation exposures are used to determine the approximate toxicity level of a chemical substance or mixture for comparative purposes. The nature of the toxic effect, if any, should also be determined through this process; in this way the concentrations to be used in subchronic inhalation exposure tests may be established. These procedures are also applicable to brief and intermittent human exposures.

The most informative and useful technique for determining the acute toxic effects of inhalation exposure is the one used to determine the LC_{50} value for rats (that atmospheric concentration statistically estimated to kill 50% of the exposed animals within a specified post-exposure period, usually four hours.) Although death is the measured endpoint for the LC_{50} determination, observation of other toxic responses should also be recorded. Test animals should be observed for at least two hours for signs of irritation of eyes, nose, and lung tissue. Gross changes in the respiratory rate, diaphragmatic breathing, gasping, and frothing and bleeding from the nares are some signs of irritation of lung tissue. Other evidence of discomfort may be pawing at the eyes or nose. In addition to these observations, records should include time of death and gross

pathological changes noted at necropsy. An acceptable post-exposure observation period is 14 days, with an optional extension to 21 days.

Exposure by inhalation is perhaps the most time-consuming and expensive of all toxicological dosing procedures. If a test substance produces a systemic toxic effect by a route of administration other than inhalation, one can assume that inhalation of a similar dosage would produce at least as great an effect (4). Thus, it may be expedient to postpone the inhalation tests or to perform them last, even though inhalation might be the most likely route of exposure. It may be possible to form a negative decision on a substance likely to be inhaled based on the results of exposure by routes other than inhalation.

The effects on the respiratory tract itself however, can be measured only by inhalation studies. These effects may be transient such as temporary inflammation, or irreversible such as death. A common direct effect is acute chemical irritation, which can affect any part of the inhalation route or the gas-exchange surfaces of the lung. These acute effects are often reversible except in cases where they produce pulmonary edema or inflammation of such severity that the lung is no longer functional (4).

It is recommended that these tests be conducted on two species of animal-the albino rat and the albino mouse. Female animals should be nulliparous. The following sample numbers and dosage schedule are recommended:

- 5 male and 5 female or 10 male animals of each species at each of two dose levels 2.0 mg/1 and 0.2 mg/1, for four hours of exposure.

ACUTE EYE IRRITATION TOXICITY

Test procedures to assess the surface toxicity of chemical substances to ocular tissues of laboratory animals should show the potential for substantial human eye injury. Albino rabbits have been most commonly used in these test procedures because their eyes are large and have no pigmentation. In addition, the tractable nature of this animal facilitates handling and examination. However, the rabbit eye differs in several anatomical and physiological respects from the human eye. The rabbit cornea is thinner, their blink reflex is not well developed, and they have nictitating membranes and thick fur on their lids.

Limited comparative data from controlled exposures of humans and rabbits show responses of the rabbit eye to be much more severe and long lasting. Other nonprimate laboratory species such as rats, guinea pigs, dogs, and cats are either less satisfactory than the rabbit or have not been thoroughly evaluated. Among nonhuman primates, the rhesus monkey has been used most frequently, but squirrel monkeys are also suitable. The potential use of monkey species as human models seems obvious, as their eyes are structurally and functionally similar to those of humans.

However, the limited availability, cost, and potential hazards in the handling of monkeys prevent their extensive use. Therefore, the albino rabbit is the species of choice, with the rhesus monkey as the preferred second species when confirmatory data are necessary.

In a given test only one eye of each animal should be used and the animal should not be subjected to extraneous test procedures or stresses. Most standard methods call for the instillation of a measured amount, usually 0.1 gm or 0.1 ml, of the test substance directly to the cornea of the test animal.

Epidemiological evidence suggests that most eye accident victims rinse their eyes with water within one minute of exposure. Certainly most physicians recommend prompt irrigation for accidental exposures to chemical substances with the rationale that the chemical on the surface is diluted and irrigated away. Some experimental animal studies indicate that irrigation may decrease the amount of irritation caused by a chemical, but is not likely to change an apparent irritant to a nonirritant. With some chemicals, such as 1% sodium hydroxide, irrigation markedly diminishes the toxic effects. With 5% sulfuric acid, irrigation exacerbates the reaction. The variability of irrigation techniques and the arbitrary nature of any one regimen further complicate this test. Consequently, irrigation is not a recommended requirement in a test of the ocular irritancy of a substance.

Interlaboratory and temporal variability in rabbit eye testing makes it difficult to determine the accuracy of any given result. Assuming that the factors that cause variability consistently affect all observations in a single test, it should be possible to compensate for them. This is done by testing control materials of established ocular irritancy and by rating unknown substances with respect to them. If the human response to the control material is known, animal response data can be extrapolated to potential human response. In such cases, the more nearly alike the test material and control are in irritancy, the more confidence can be placed in the extrapolation.

If healing of the cornea and conjunctiva follow chemical injury, it is usually completed within 14 days. Therefore, observation for 21 days is essential in any test for toxicity. The recommended times for observations are 1 hour, 1, 3, 7, 14, and 21 days, though slight deviations from this schedule will not seriously affect results.

Scoring the results of ocular irritancy tests is complex and several scoring systems have been developed to assess the degree of damage. Observations of the cornea, iris, conjunctivae should be reported as well as serious lesions such as pannus, phlyctena, and rupture of the eyeball. The grades of ocular reaction must be recorded at each examination. Evaluation of these reactions can be facilitated by use of a binocular loupe or hand slit-lamp.

At the 24 hour observation, the eyes of any or all test animals may be further examined after applying fluorescein stain. For this optional examination, one drop of fluorescein solution is dropped directly on the cornea. After flushing out the excess fluorescein with tap water or saline solution, the injured areas of the cornea will appear yellow in ultraviolet light.

A record of the discharge from treated eyes in not required; however, any exudate above normal can be recorded as additional information.

In grading these tests, an animal has exhibited a positive reaction if the test substance has produced one or more of the following signs at any observation:

a) Ulceration of the cornea beyond a fine stippling effect

b) Opacity of the cornea, other than a slight dulling

c) Inflammation of the iris, other than a slight deepening of the rugae or a slight hyperemia of the circumcorneal blood vessels

d) Swelling in the conjunctivae (excluding the cornea and iris) with partial eversion of the eyelids and a diffuse crimson color with individual vessels not easily discernible.

In Table 2 a grading system for these parameters is presented. Frequency counts are then made for each ocular parameter and Table 3 can be used to aid in labelling the test substance.

The test should be considered positive if four or more animals exhibit a positive reaction. If only one animal exhibits a positive reaction, the test should be regarded as negative. If two or three animals exhibit a positive reaction, the substance is considered to be an irritant.

A clear distinction must be made between those substances that produce transient irritation and those that produce substantial injury. Substances that produce conjunctivitis which clears within 2-3 days with no further reactions generally require no further investigation. Materials that produce serious corneal injury or internal injury to the eye are serious hazards and appropriate controls should be placed on their use. It is also important to note any signs of systemic toxicity in the course of these tests.

The recommended test species are the albino rabbit and albino rat. The suggested sample numbers and dosage are:

6 animals of each test species at one dose of 0.1 gm (or 0.1ml) in a single eye of each animal.

TABLE 2

Grades for Ocular Lesions

CORNEA

No ulceration of opacity.....0
Scattered or diffuse areas of opacity.....1
Translucent areas, details of iris slightly obscured.....2
Nacreous areas, no details of iris visible, size of pupil barely discernible.....3
Opaque cornea, iris not discernible.....4

IRIS

Normal.....0
Markedly deepened rugae, congestion, swelling or circumcorneal hyperemia, but iris still reacting to light.....1
Hemorrhage, gross destruction, or no reaction to light.....2

CONJUNCTIVAE

Blood vessels normal.....0
Some blood vessels hyperemic.....1
Diffuse, crimson color, individual vessels not easily discernible.....2
Diffuse, beefy red color.....3

LIDS AND/OR NICITATING MEMBRANES

No swelling.....0
Slight swelling.....1
Moderate swelling with partial eversion of lids.....2
Swelling with lids about half closed.....3
Swelling with lids more than half closed.....4

TABLE 3

Classification of Test Substances

Classification		Ocular Reaction
Non-irritant	--	No positive reaction in any category in more than 1 out of 6 test animals at 1-3 days and all eyes normal at 7th day.
Irritant	--	Corneal opacity grades of 1.0 to 2.0 at any observation up to 7 days, but all corneas cleared by 14th day.
	--	Iritis grades of 1.0 at 1-7 days, but all iritis cleared by 14th day.
	--	Conjunctivitis grade of 2.0 or more at 1-7 days.
	--	Lid or nictitating membrane swelling grades of 2.0 or more at 1-7 days.
Severe Irritant	--	A positive reaction in any category which has not cleared by 14 days.

DERMAL IRRITATION TOXICITY

A reliable test for skin irritation should provide a means for differentiating among substances that will produce different degrees of irritation or corrosion of the skin. In this context, irritation is the local inflammatory response of normal living skin to direct injury by single, repeated, or prolonged contact with a chemical agent without the involvement of an immunologic mechanism. The macroscopic manifestations are erythema and edema. Corrosion is direct chemical action on normal living skin that results in its disintegration and irreversible alteration at the site of contact. Its important manifestations are ulceration, necrosis, and with time, the formation of scar tissue. It is especially important to be able to distinguish between materials that will produce minor or inconsequential degrees of skin irritation from materials that can produce substantial irritation or corrosive injury as a result of either customary use or accidental exposure.

The voluminous literature on primary irritation test methods lacks consensus on the animal model or procedure most likely to give accurate and dependable results. Test procedures for human subjects are as numerous as those for animals, suggesting that the problem does not lie solely in selection of the test species. The most standardized procedure is a 24-hour, semiocclusive patch test of a full-strength substance on the skin of albino rabbits. A common test procedure is to demarcate about 100 cm^2 of the skin into quadrants - two abraded and two intact. The test substance can then be applied to one abraded and one intact quadrant, leaving another quadrant of each type to serve as controls. Most of the conventional laboratory animals and some of the more exotic species have been tried in skin irritancy testing. None provide perfect models for human skin. The albino guinea pig and albino rabbit, though commonly used, lack the broad spectrum of human responses to skin irritants. They show only degrees of erythema and edema. Both species react more strongly than humans to mild-to-moderate irritants. In fact, some materials that appear unsafe when tested on rabbits may be nonirritating to human skin. The response to guinea pig skin is more like that of human skin over a wide range of materials; thus it is preferable to the rabbit.

The chemical substance can be applied to intact and abraded test sites on clipped animals for periods of exposure that range up to 24 hours. A statistically significant number of test sites (usually 2-4 sites on each of six animals) should be available for evaluating each substance.

After removal of the test substance, 30-60 minutes should lapse before the patch sites are read to allow sufficient time for pressure and hydration effects to subside. Additional readings should be made 24 and 72 hours after the patch application. Some persistent effects such as corrosion are better determined at 7 days.

Evaluation of skin effects involves using a scoring system for the degree of redness and the degree of edema at the site of application of the test substance. The scoring system usesd is commonly that which has been published in the Federal Register in Section 191.11 of the Federal Hazardous Substances Act of the United States. Their scoring system, as seen in Table 4, involves assignment of numbers for the relative degree of erythema and the degree of edema formation.

TABLE 4. ERYTHEMA AND EDEMA SCORING

Erythema

0 = no erythema
1 = slight, barely perceptible erythema
2 = well defined erythema
3 = moderate to severe erythema
4 = severe, beet red erythema with injuries in depth

Edema

0 = no edema
1 = very slight, barely perceptible edema
2 = slight edema with raised edges
3 = moderate edema with surface raised approximately 1 mm
4 = severe edema with the area raised more than 1 mm and extending beyond the area of exposure

The scores obtained for both erythema and edema at each scoring period and for both the abraded and intact skin are listed and the mean for each group and for each type of effect is calculated. All 8 mean values are then added together and divided by 4 since there are four mean values for each effect (erythema and edema) thereby giving a final numerical figure which is the primary irritation score.(5)

In some cases it may be useful to retain animals for 2 weeks after application but because such delayed readings usually only confirm effects seen at 7 days, the value of further observation should be measured against the cost of maintaining the animals.

Table 5 reflects the kinds of warnings which can be developed from the numerical results of these tests.

The suggested test species are the albino rabbit and the albino rat. Recommended sample numbers and dosage schedule are:

6 animals of each test species at 100%, 30%, 10%, 1%, and 0.3% of the test compound. If the lowest of these produces positive results, a dose below 0.1% is used to find a no-effects level.

TABLE 5
NATIONAL INSTITUTE FOR OCCUPATIONAL SAFETY AND HEALTH
INTERPRETATION OF SKIN TEST RATINGS (6)

	Rating	Interpretation
Intact skin	0-0.9	Nonirritant; probably safe for intact human skin contact
	1-1.9	Mild irritant; may be safe for use, but appropriate protective measures are recommended during contact
	2-4	Too irritant for human skin contact; avoid contact
Abraded skin	0-0.9	Nontoxic to cellular components of abraded skin; probably safe for human skin contact
	1-1.9	Mild cellular toxins; may be safe for abraded skin contact provided protective measures are employed
	2-4	Cellular toxins too irritant for abraded skin contact; avoidance of contact is advised

Mixed reactions		
Intact skin	Abraded skin	
0-0.9	0-0.9	Safe for human skin contact
	1-1.9	Safe for intact human skin contact; may be safe for abraded skin contact when protection is maintained
	2-4	Safe for intact human skin; contact with abraded skin should be avoided
1-1.9	1-1.9	May be safe for intact and abraded skin contact when protection is maintained
	2-4	May be safe for intact human skin contact when protectio is maintained, but contact with abraded skin is to be avoided
2-4	2-4	Unsafe for intact and abraded human skin contact; avoid contact

DERMAL SENSITIZATION TOXICITY

Dermal contact sensitization refers to a delayed, immunologically-mediated, allergic reaction to a chemical. With few exceptions, contact sensitization develops as a result of one or more contacts with a chemical which initiate the sensitization process. The latent sensitized condition generally develops no sooner than 1-2 weeks after the effective exposure. Subsequent exposure of the skin of the sensitized individual to a lower concentration of the sensitizer or related substance (cross-sensitizer) can elicit a more intense response than to the initial exposure to the chemical. This response may take hours or even days to develop, hence it is "delayed." Responses may be characterized by pruritis, erythema, edema, or induration, papules, vesicles, bullae, or combinations of these. Reactions generally subside over a period of days if there is no further contact with the sensitizer, but the state of sensitization may be permanent.

Occasionally an individual who has been sensitized through the skin will exhibit a systemic reaction, anaphylactic shock, whose symptoms include irritability, dyspnea, cyanosis, convulsions, unconsciousness and death.

Dermal sensitization tests should detect materials that are capable of inducing either a substantial incidence of or degree of sensitization responses among individuals exposed during use or accidental misuse.

Laboratory animal species are generally much less responsive to contact sensitizers than are humans. The most responsive is the guinea pig, particularly albino varieties. Animals from 1-3 months of age are preferred as they are more easily sensitized than very young or older animals. There is no appreciable difference in the proclivity of male and female guinea pigs to develop sensitization, but pregnant females should be avoided since pregnancy may alter the allergic response.

Topical application techniques for the determination of skin sensitization have been in common use for the last twenty years. A method which involves intradermal injection of the test material in guinea pigs is also used, although this technique does not represent true topical sensitization.

This testing involves both an induction phase and a challenge phase. The concentrations of test compound to be used in the induction phase as suggested by Klecak (7) are 100%, 30%, 10%, 3%, 1% and 0.3%. On the first day of testing, a 0.1ml solution or suspension of each of these

concentrations of the test substance is applied to an area measuring 8 cm^2 on the clipped flank skin of six to eight animals per concentration group. Applications are repeated daily for 3 weeks, or five times weekly for 4 weeks, always using the same skin site. The application site remains uncovered and the reactions (if continuous daily applications are performed) are read 24 hours after each application or at the end of each week. When strong local irritation reactions are elicited, the application site is changed. A control group of six to eight animals is not induced.

The concentrations to be used in the challenge phase will have been determined largely from the results of the dermal irritation studies. Each test animal and each control will be challenged by each of four concentrations of the test substance:

- the minimal irritating concentration (the lowest dosage at which 25% of the test animals develop a mild erythema, an erythema score of 1, but no edema.),
- the maximum nonirritating concentration (the highest dosage which elicits no skin reaction), and
- two scaled doses below the maximum nonirritating concentration

On days 21 and 35, applications of 0.025 ml of each of these four concentrations are applied to the contralateral flank of each animal on a skin site measuring 2 cm^2. Reactions are read at 24, 48, and 72 hours. This procedure determination of the minimal sensitizing concentration necessary for inducing allergic contact hypersensitivity and the minimal eliciting concentration necessary to cause a positive reaction. A concentration is considered allergenic when at least one of the animals in that concentration group concerned shows a positive reaction with nonirritant concentrations.

Because cutaneous responses are visible, they can be readily evaluated by a trained observer. The delayed reactions of contact sensitization are best evaluated by making sequential observations of test sites on the skin. The area is scored for the degree of erythema and edema as described above in the dermal irritation section. Numerical scores are then averaged for the animals in each group. The initial observation should be made 48 hours after the first application of the test mixture and these scores are used as an index of the irritant properties of the substance. A second observation should be made 24 hours after the challenging injection in order to allow primary irritation to subside, but subsequent scorings should be made 48 hours later after the challenging dose. The scores that are taken following the challenging dose are compared with the irritant scores. If the challenge scores are two to four times the irritant scores the compound is considered to be a mild sensitizer. Compounds that have high sensitizing activity will show sensitizing scores that are four to seven times the irritant scores. (8)

Reactions to the test substance at challenge that are stronger than reactions to negative controls or to those seen during induction should be suspected as results of sensitization. Responses that are marginally more intense than control response or that occur in very few animals should be confirmed by a second challenge after 1 or 2 weeks. Rechallenge after a longer delay can produce unreliable results, because sensitization in guinea pigs is short-lived compared to that in humans. Whether or not a rechallenge is performed, a judgement confirming the presence or absence of sensitization should be recorded for each animal. Mean scores or indices, which are customarily calculated for each experimental group, are useful only for showing relative intensity of response. Two or more unequivocally positive responses in a group of 10-20 animals should be considered significant. A negative, equivocal, or single response probably assures that a substance is not a strong sensitizer, though this is best confirmed by further testing with human subjects.

The albino guinea pig and the albino rat are suggested as the test species and the following sample numbers and dosage is recommended:

> 5 male and 5 female animals per concentration group each induced with 0.1 ml of the test substance at 100%, 30%, 10%, 3%, 1%, 0.3% or 0% (controls). Each animal is then challenged with 0.025 ml applications of the test substance at four concentrations as determined from the dermal irritation studies: 1) the minimum irritating concentration, the maximum non-irritating concentration, 3 & 4) two scaled does below the maximum non-irritating concentration.

PHOTOTOXICITY

A phototoxic response refers to irritation that is not immunologically-mediated and which depends on light exposure for its presence. This response is not to be confused with photosensitization or the photoallergy response in which light energy results in the promotion of hapten formation and the consequent appearance of antigen. In this case it is the resultant antigen-antibody response that is the cause of cell damage. At this point neither the need nor the exact protocol requirements have been established for photosensitization testing, thus it is not presently recommended at this level of acute testing.

Responses to phototoxic chemicals are elicited by ultraviolet light wavelengths, those between 280 and 430 nm. The agent plus light energy results in the excitation of the agent; its energy state is raised to a higher level. In the process of returning to its original ground state of energy, the compound can react with constituents of the cell. Although the mechanism of action of phototoxicity is not clear, the chemicals which elicit this reaction are thought to be free radical initiators. These compounds can react with molecular oxygen in the presence of light energy to form peroxides that cause damage to cell membranes.(8)

The chemical to be tested is administered in a solvent which will not alter the test situation by reacting with the chemical or otherwise absorbing energy upon exposure to ultraviolet light. Dosing should be on a microgram or milligram per square centimeter basis, simplifying the extrapolation to dosing in humans. The skin site can be conveniently demarcated with a marking pen and the chemical can be delivered to the skin with a micropipette. Following application, the animals are exposed to ultraviolet light from a high output source.

It is customary to administer one high dose of the test substance. This may be up to 10% of the oral LD_{50}. No situation has yet been found in which a compound has been negative at a high dose and positive at a low dose. If the high dose elicits a positive response, the least effective dose is then determined. Each animal may be used as its own control. Control sites include 1) negative (the vehicle), 2) positive (a known phototoxic chemical), and 3) unirradiated, chemical-treated sites. Groups of 4-10 animals, are sufficient for this testing.

The phototoxic response is usually elicited quickly. For maximum effect the site should be irradiated within 30 minutes to 2 hours after the chemical application. Scoring as described for dermal irritation tests is performed 12-24 hours later. The result most commonly found in phototoxic responses is a visible and palpable dermatitis consisting of erythema, induration, and at times, frank necrosis. The phototoxic response is dramatic; there are few tests easier to read. Certain phototoxic chemicals and solvents may irritate the skin without irradiation. When this occurs, attempts should be made to decrease the effective tissue dose so that the irritancy is not seen at the site where the chemical has been applied but has not been exposed to light.

Occasionally, extrapolation of results obtained from animal phototoxicity tests to humans may be questionable. In such cases, tests with humans may be necessary if the basic systemic toxicololgic data are available. The experimental procedure resembles that used with animals; however, it is usually necessary to make human skin more permeable to the test substance by removing most of the stratum corneum by repeated cellophane tape stripping. A stripped skin site control is also used. The dose should be administered in one small application.

ORAL IRRITATION TOXICITY AND CORROSIVE EFFECTS

It is particularly important to be able to detect materials which, if ingested, can produce corrosive injury to the mucosal surfaces of the oral cavity, pharynx, esophagus, and stomach. Severe corrosive injury to these tissues can be fatal or can result in strictures or other permanently disabling injuries. Chemical properties are reliable predictors of corrosive potential. Strong alkalis are likely to injure the esophagus and strong acids to injure the stomach and duodenum. Either may injure the tongue and pharynx.

There is no standardized procedure for predicting corrosive potential to the alimentary tract, though several techniques have been described. Materials have been admininstered by intraoral, intraesophageal, and intragastric gavage and by timed application to specific tissues by solutions or impregnated tampons. Rats, rabbits, cats, dogs, and swine have been tested, but a preferred animal model has not been identified. The experts agree that more research is needed before an animal model is selected and a reliable procedure is established.

The need for a special test for esophageal corrosivity has been questioned on the grounds that information on the chemical and physical properties of a substance, as well as the results of dermal irritation studies, can provide reasonable presumptive evidence of severe irritation or corrosive hazard upon ingestion even in the absence of specific corrosivity testing.

SECTION 3

ACUTE TOXICITY TESTING CRITERIA

WHEN SHOULD ACUTE TOXICITY TESTING BE PERFORMED?

It is recommended that all chemicals substances be screened for acute toxicity and their LD_{50}s be determined. It has been suggested that certain substances should not be required to undergo testing for toxicological effects including those whose physical and chemical properties and structure-activity relationships indicate that no adverse health effects are probable.

Examples of this situation include:

- nonreactive insoluble polymers which pose no potential human exposure through inhalation of particles in air
- nonreactive insoluble polymers which contain no monomeric contaminants or have them at levels safely below those known to cause human toxicity
- new chemical substances which differ only slightly in structure from known substances which have already been shown to be innocuous by existing toxicity data.

These may be reasonable exemptions in theory but in practice, biological nonreactivity should be demonstrable and not merely assumed. It is difficult to assure "safe" levels of monomeric contaminants in polymers without first measuring those levels and evaluating their toxicity. It is difficult to define a "slight" modification in structure. This is a very ambigious exemption and may ultimately be abused. Structural-functional relationships may be useful predictors but it is also true that small differences in chemical structures can significantly influence the biological effects of chemicals.

A common example is found in the compounds benzene and toluene. Benzene is associated with severe acute toxicity including irritation of the mucous membranes, convulsions, and respiratory failure. Chronic effects include bone marrow depression, aplasia, and occasionally leukemia. Toluene, on the other hand, which differs structurally from benzene only by the addition of a methyl group, is considerably less toxic than benzene. Toluene at high concentrations may cause mild macrocytic anemia but it is not associated with the broad spectrum of hematologic toxicity which is seen with benzene exposure.

Another example is found in the drug amphetamine (racemic -phenylisopropylamine) whose effects include central nervous system stimulation and activation of receptors which are normally innervated by the sympathetic nervous system. The d-isomer is three to four times more

potent than the l-isomer in its effects on the central nervous system whereas the l-isomer is twice as effective as the d-isomer in its effect on the heart. An effect of valence number on the toxicity of arsenic can also be seen. The trivalent arsenites are much more lethal than are the pentavalent arsenates for lower plants and animals including protozoa, bacteria, and yeast(8). In essence, most predictions about toxicity and structural-functional relationships should be verifiable and not simply left as assumptions.

Testing exemptions may be considered for minor alterations to sites other than the reactive group on such compounds as epoxides, nitrosamines, and nitrosoureas. But here too there must be some consensus as to what changes would have no effect on the biological reactivity of the molecule. In these cases it will be easier to implicate a compound as toxic than to exonerate it as safe.

Another group of compounds that has been suggested as possible exemptions from toxicological testing includes those in which adverse health effects are anticipated but human exposure will be strictly controlled. This includes:

- substances which are extremely acidic (pH <2) or basic (pH >13), which are not volatile, which are used solely for industrial purposes, and for which human contact would be strictly prevented by protective measures.

Compounds of extreme acidity or basicity in their industrial stages may remain as trace residues in or on some resultant product intended for human use. At this point corrosivity may no longer be a factor but other toxic properties might possibly become evident. Exemptions to substances in this category do not seem to be justified unless it can be shown unequivocally that detectable levels never remain in the products to which humans may ultimately be exposed.

WHAT ACUTE TOXICITY STUDIES SHOULD BE CONDUCTED:

The first level of testing should provide enough information to be suggestive of the need for further testing in any area of toxicity and should also be as simple, fast, and inexpensive as possible. It is apparent that complete testing for long-term, irreversible effects will not be required for all substances, and thus should not be included in the first level of tests. It seems prudent to require a complete battery of acute toxicity tests in the initial testing stage in order to obtain useful data and possibly give some indication of the need for subchronic testing as a second series of tests.

Acute toxicity tests recommended are as follows:

- <u>acute oral toxicity</u> - for all chemical substances except gases

- acute dermal toxicity - for all chemical substances except gases

- acute inhalation toxicity tests - for those substances whose physical nature suggests that they would be airborne and thus where inhalation is expected to be a possible route of human exposure including:

  - all gases

  - liquids with a vapor pressure higher than 1 torr

  - solids with a particle size below 5 micra in diameter

- Irritation and sensitization tests - for all chemical substances except gases.

WHAT ARE THE MINIMAL PROTOCOLS FOR THE ACUTE STUDIES?

Several studies have examined the variability of acute toxicity test results (specifically the LD_{50} calculations) obtained on substances examined in different laboratories. Even when the same testing procedure was followed in each of eight laboratories, LD_{50}'s differed by up to 5-fold; averaging a 2-3-fold difference between highest and lowest values. This indicates that general guidelines for protocols would be more appropriate than rigid procedures for acute toxicity studies.

Acute toxic effects in animals can range from no observed effect to sedation, loss of motor function, loss of consciousness, respiratory depression, and death. The intensity of effect is related to time, and also to blood concentration. Thus, there are two major factors in acute toxicity tests: the rate of absorption and the rate of elimination of the test substance. These two rates are in turn influenced by several factors, including:

- physical/chemical properties of the substance
- concentration
- volume
- route of admininstration
- biotransformation
- transport
- excretion routes

- species (age, sex)
- pre-existing pathology.

These various influencing factors will not be identical between laboratories, or even within the same laboratory at different times.

In general, it would help to check on the testing laboratory's accreditation as at least a general indication that the test was conducted in accordance with good laboratory practices.

In toxicity testing a primary consideration is the purity of the substance to be tested. The purity of the test substance should be known and any contaminating components should be identified and quantitated as thoroughly as possible to account for 100% of the test substance. All materials should be stored appropriately to maintain their purity and chemical integrity. Whenever possible, all studies should be done using the same lot of test sample.

It is suggested that for each type of acute test to be performed, two species of test animal should be chosen, and one of these should be the albino rat. This will allow comparison of the effects of a given chemical substance administered to the same animal model by all test routes. Correlations can then be attempted between oral, dermal, and inhalation LD_{50}'s. Rats are preferred also because there is abundant reference toxicologic data available for this species.

The second species should be the albino rabbit in tests of dermal toxicity, dermal irritation and eye irritation; the albino guinea pig should be used in tests of dermal sensitization; the albino mouse would probably suffice as the second species in tests of oral and inhalation toxicity.

In order to standardize results within and among laboratories, all protocol requirements should be as specific as possible. Therefore, if the general test animal is to be the albino mouse, an example of the specific requirement would be:

> C57Bl/C3H mice (F-1) from Caesarean-derived animals, maintained free from Mycoplasmosis (CRD) Salmonella, external parasites, internal parasites, and other disease are to be used. The supplier should provide information about the type of diet the animals have been fed and their bedding. Arrange animal delivery in time to be ready for assignment to treatments at about 42 days of age.

Dietary requirements should also be carefully delineated. Any diet which meets the general nutritional requirements of the species in question can be used as a standard diet in toxicity studies so long as the exact formulation and processing of the diet are known. For standardization among testing agencies, the NIH Interagency Toxicology Support Committee has recently recommended use of the NIH-7 Open Formula Rat and Mouse Ration (Table 6) as a standard diet. This formula is available from several manufacturers and is priced competitively with other commercial feeds. It is suggested that this be the required diet in all studies using rodents as the test animal.

Caging requirements and animal care procedures should also be very specific since these features can influence not only reproducibility of experimental results but can also affect worker safety. A suggested requirement would be:

> Test animals should be housed as a single animal per cage in hanging stainless steel wire cages. The use of wire mesh bottoms without bedding is preferred since this will prevent coprophagy and induction of mixed function oxidases. However, closed housing with false wire cage bottoms may be used if precautions are taken to prevent coprophagy.

It is recommended that animal care during these studies include the following standard requirements:

- Temperatures will be kept at 27-29°C with a 12 hour dark-12 hour light cycle.
- Clean drinking water will be provided and changed twice weekly.
- Food consumption and body weights will be recorded at weekly intervals.
- Cages and racks will be cleaned and sanitized once every two weeks.
- Floors will be sanitized daily.
- Animals will be observed daily by trained animal care personnel.
- Sick or dead animals will be removed for complete necropsy, after notification of the investigator or his representative.
- Proper records will be kept daily.
- Facilities and equipment will be monitored for sanitation on a monthly schedule.

TABLE 6, NIH-7 OPEN FORMULA RAT AND MOUSE RATION[1]

Ingredient	%
Dried skim milk	5.00
Fish meal	10.00
Soybean meal	12.00
Alfa'fa meal	4.00
Corn gulten meal	3.00
Ground No. 2 yellow shelled corn	24.50
Ground hard winter wheat	23.00
Wheat middlings	10.00
Brewers dried yeast	2.00
Dry molasses	1.50
Soybean oil	2.50
Sodium Chloride	0.50
Dicalcium phosphate	1.25
Ground limestone	0.50
Vitamin and Mineral Pre-mixes[2]	0.25
	100.00

[1]Calculated approximate composition = crude protein, 23.5%; crude fat, 5.0%; crude fiber, 4.5%; ash, 7.0%.

[2]Vitamin and mineral pre-mixes shall provide per kg diet: vitamin A (stabilized), 6,050 IU; vitamin D_3, 5,060 IU; vitamin K, 3.1 mg; a-tocopheryl acetate, 22 IU; choline 0.6 g; folic acid, 2.4 mg; niacin, 33 mg; d-pantothenic acid, 20 mg; riboflavin, 3.7 mg; thiamin, 11 mg; vitamin B-12, 4.4 µg; pyridoxine, 1.9 mg; biotin, 0.15 mg; cobalt, 0.44 mg; copper, 4.4 mg; iron, 132 mg; manganese, 66 mg; zinc 18 mg; iodine, 1.5 mg.

Another important consideration is the gross pathological examinations that are required. It is suggested that the following requirements be considered for adoption:

> Any animal which dies in the course of the study must be refrigerated at temperatures low enough to minimize autolysis if necropsy cannot be performed immediately. Necropsy must be performed on all animals who die or who are sacrificed within 24 hours and preferably within 16 hours of death. The gross dissection and evaluation shall be performed by or under the supervision of the pathologist.
>
> The necropsy is defined as an external examination, including body orifices, and examination and fixation of the following tissues:
>
> Gross lesions
> Tissue masses or suspect tumors & regional lymph nodes
> Skin
> Mandibular lymph node
> Mammary gland
> Salivary gland
> Larynx
> Trachea
> Cecum
> Colon
> Rectum
> Mesenteric lymph node
> Liver
> Thigh muscle
> Sciatic nerve
> Sternebrae, vertebrae, or femur (plus marrow)
> Costochondral junction, rib
> Thymus
> Gallbladder
> Pancreas
> Lungs & bronchi
> Heart
> Thyroids
> Parathyroids
> Esophagus
> Stomach
> Duodenum
> Jejunum
> Ileum
> Spleen
> Kidneys
> Adrenals
> Bladder
> Seminal vesicles
> Prostate
> Testes
> Ovaries
> Uterus
> Nasal cavity
> Brain
> Pituitary
> Eyes
> Spinal cord
>
> Specific requirements for gross necropsy procedures, and pathology reports are described in NCI publication CG-TR-1 which might be distributed to the test laboratories. Histopathological examinations are considered to be too costly for this stage of testing and are not expected to reveal significantly more information than gross pathology reports.

Estimated costs for each test will vary with time and the location of the laboratory. An example of the costs of various acute toxicity tests is shown in Table 7.

TABLE 7, ACUTE STUDY PRICE LIST
(July, 1979)

EYE IRRITATION $390.00

6 rabbits
72 hour evaluation

EYE IRRITATION $510.00

9 rabbits
6 unwashed
3 washed
1 week evaluation

PRIMARY SKIN IRRITATION $360.00

6 rabbits
14-day evaluation

ACUTE ORAL TOXICITY $900.00

Five levels of ten rats/level
72 hour evaluation

ACUTE ORAL TOXICITY $325.00

Six levels of two rats/level
72 hour evaluation

ACUTE DERMAL TOXICITY $850.00

Two levels of ten rabbits/level
14 day evaluation

ACUTE DERMAL TOXICITY $1050.00

Four levels of four rabbits/level
14 day evaluation

ACUTE DERMAL TOXICITY $3400.00

Five levels of eight rabbits/level
14 day evaluation

ACUTE ORAL TOXICITY $900.00

Five levels of ten mice/level
14 day evaluations

Table 7 continued

SENSITIZATION $1200.00

One level of ten guinea pigs/level
Five week evaluation

SENSITIZATION $2300.00

Two levels of ten guinea pigs/level
Five week evaluation

HOW SHOULD THE RESULTS BE INTERPRETED AND USED TO ESTIMATE A TOLERABLE LEVEL?

In order to extrapolate the results of acute tests performed on laboratory animals to "safe" levels of chronic human exposure, certain obvious considerations are necessary. One such consideration is the relationship of animal toxic responses to human toxic responses and the other is the relationship of short-term high-dose toxicity to long-term low-dose toxicity.

A convenient approach to the question of the animal-human sensitivity differential is to use the hundredfold safety margin. This is based on a convention which assumes that humans may be up to tenfold more sensitive than the experimental animals used and that additonally there may be a tenfold variation in sensitivity among individuals.

The second consideration, extrapolation of data from short-term high-dose studies to predict the outcome of long-term low levels of exposure, is a more complex problem. Several noted toxicologists have stated that any acute toxic sign that will be produced at a given dose will occur within 90 daysof the initial administration of the test substance. (9-17) Also, the results from two major studies have indicated that lifetime no-effect doses can be predicted from short-term studies.

The Weil and McCollister study (18) determined the 3 month and 2 year no-effect doses of 33 compounds of diverse chemical structure, pharamcologic type, and toxicity in rats and dogs. These compounds included pesticides, veterinary chemicals, food additives, thickeners, stabilizers, antimycotics, and water treatment substances.

The animals were observed for 36 criteria of toxicity including mortality, food intake, weight, pathology, blood chemistry, hematology, cholinesterase, fertility, and neoplasia. The most sensitive criteria were body weight, ratios of liver and kidney weight to body weight, and kidney pathology. It was the feeling of these authors that only these sensitive parameters need to be followed.

There were various numerical relationships by which the long-term no-effect dose for each compound could be derived from its short-term no-effect doses. But in 97% of the compounds tested (or 32 out of 33) it was seen that 1/10 of the 3 month no-effect dose could be given repeatedly throughout a lifetime without producing toxic effects; 100% of these compounds were non-toxic given at 1/12 of the 3 month no-effect dose.

This approach is further supported by a recent literature survey by McNamara (19) in which data was collected on the short-term and long-term no-effect doses of 122 compounds of diverse chemical and pharmacological

types. These studies were conducted in various laboratories using different methods and animal species. The resulting frequency distributions of the LD_{50} levels and short term and long term no-effect doses demonstrated three basic relationships:

- any compound administered to an animal at one hundredth of its LD_{50} will produce no effects in a short-term (3 month) study
- any compound administered daily at one tenth of its 3 month no-effect dose will produce no effects in the lifetime of that animal
- any compound administered daily at one thousandth of its LD_{50} will produce no effects in the lifetime of the test animal.

Using the third observation, we can use the LD_{50} derived from short term acute testing to predict the lifetime NOEL (no observable effects level) within the same animal species.

The lifetime NOEL of the test animal may be further modified, as described above, to accomodate the human-animal sensitivity differential. The lifetime NOEL for the test animal divided by 100 becomes the proposed TLE (tolerable level of exposure) for humans.

The working hypothesis therefore becomes:

> The LD_{50} derived from short term animal studies divided by 100,000 is the TLE of chronic human exposure.

This safety factor of 100,000 is very conservative and its use is most justified when the results of acute testing produce a flat dose-response curve or a delay in the onset of and recovery from toxic signs. These data may be suggestive of a potential for cumulative toxicity and thus the need for longer term testing (1). Physical/chemical properties of the toxicant may also indicate the need to apply a greater safety factor when determining a NOEL in humans.

In other situations, where a less conservative approach seems to be justified, one might reduce the human-animal sensitivity differential to 10 and use the LD_{50} from animal studies divided by 10,000 to predict the tolerable level of chronic human exposure.

An example of how one might use each of these factors is demonstrated in Table 8 using some oral LD_{50}s from the literature(21):

TABLE 8. PREDICTIONS OF HUMAN TLEs FROM ORAL LD_{50}s DERVIED FROM ANIMAL STUDIES

Compound	Species	Oral LD_{50} (mg/kg)	Predicted Human TLE[a] (g/kg day)	Conservative Predicted Human TLE[b] (g/kg/day)
Methyl Salicylate	dog	2100	210	21
Thiabendazole	rat	3100	310	31
Propylene glycol	rat	27000	2700	270
Pimaricin	rat	3700	370	370

a. figures given are oral LD_{50}s divided by 10,000.
b. figures given are oral LD_{50}s divided by 100,000.

HOW SHOULD THE DATA BE SUBMITTED FOR EASY INTERPRETATION BY REVIEWERS?

All dose and response data should be presented in tabular form. Doses should be calculated in mg/kg body weight. Responses should be listed as number of animals killed per number of animals exposed in each dosage group. Other toxic effects should also be noted and recorded for each dosage level group.

It is suggested that manufacturers perform their own calculations of dose-response relationships, slopes, standard deviations, and LD_{50}s, the use of a second party to observe and monitor these calculations is also recommended. Simple computer programs can be used to facilitate operations and minimize errors.

Experimental protocols should also be fully described by each laboratory in order to check for any procedural errors which could influence the accuracy of the data.

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